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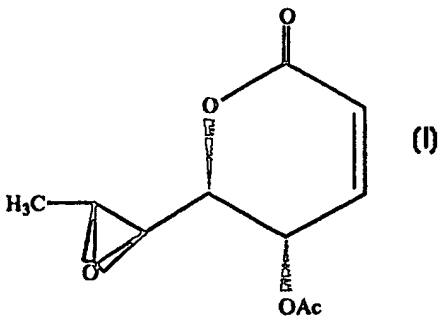
(57) Abstract

This invention concerns compositions and methods utilizing a compound of formula (I) for reducing or controlling inflammation and treating pathological conditions mediated by intercellular adhesion. More particularly, the present invention concerns compositions and methods for blocking or modulating the function of the Beta$_2$ Integrin family of cell adhesion molecules.



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BETA$_2$ INTEGRIN CELL ADHESION MOLECULE INHIBITORS

This invention concerns compositions and methods for reducing or controlling inflammation and treating pathological conditions mediated by intercellular adhesion. More particularly, the present invention concerns compositions and methods for blocking or modulating the function of the Beta2 Integrin family of cell adhesion molecules.

Background of the Invention

Stanley P. Owen and B.K. Bhuyan describe the isolation of the crystalline antibiotic 2H-Pyran-2-one,6-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy acetate, which they refer to as U-13,933, in their article "Biological Properties of a New Antibiotic, U-13,933", Antimicrobial Agents and Chemotherapy-1965, copyright 1966, pp. 804-807.

A.D. Argoudelis and J.F. Zieserl described further structural specifications of the antibiotic U-13,933 in "The Structure of U-13,933, A New Antibiotic", Tetrahedron Letters No. 18, pp. 1969-1973, 1966.

U.S. Patent No. 3,909,362 (Jiu et al.) discloses and claims a process for the production of the antimicrobial agents 5,6-dihydro-5(S)-acetoxy-6(S)-(1',2'-trans-epoxypropyl)2H-pyran-2-one, 5,6-dihydro-5(R)-acetoxy-6(S)-(1',2'-trans-epoxy-propyl)2H-pyran-2-one, and 5,6-dihydro-5(S)-acetoxy-6(S)-(1',2'-trans-propenyl)2H-pyran-2-one comprising growing *Aspergillus sp.* NRRL 5769 or *Aspergillus sp.* NRRL 5770 in an aqueous nutrient medium containing sitosterol or sitostenone and isolating the compounds from the medium.

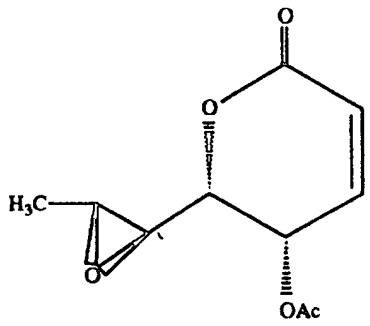
The three metabolites taught in the Jiu et al. patent, above, were further explained as showing antimicrobial activity against *C. albicans*, and against specific bacteria, fungi and a trichomonad by S. Mizuba et al. in "Three antimicrobial metabolites from *Aspergillus caespitosus*", Can. J. Microbiol., Vol. 21, 1975, pp. 1781-1787.

In their article "Total Synthesis of (+)-Asperlin", Tetrahedron:Assymetry Vol. 1, No. 3. pp. 137-140, 1990, Subban Ramesh and Richard W. Franck describe a

stereochemically unambiguous synthesis of (+)-asperlin, a crystalline antibiotic from *Aspergillus nidulans*, from L-rhamnose and cite the configuration of the antibiotic as 4S, 5S, 6S, 7R.

Brief Description of the Invention

The present invention comprises compositions and methods for blocking or modulating the function of the Beta2 Integrin family of cell adhesion molecules in a mammal, preferably in a human, the compositions and methods utilizing the compound having the structure:



which is named 6,7-Anhydro-2,3,8-trideoxy-D-galacto-oct-2-enoic acid.delta.-lactone 4-acetate, also referred to as 5,6-dihydro-5(S)-acetoxy-6(S)-(1,2-trans-epoxypropyl)-2H-pyran-2-on.

Detailed Description of the Invention

The present invention includes pharmaceutical compositions and methods of administering to a mammal, preferably to a human, the compound of this invention to inhibit intercellular adhesion mediated by the β_2 Integrin family of cell surface molecules. Through this inhibitory activity the pharmaceutical compositions and methods of the present invention are useful in treating or inhibiting inflammatory and other pathological responses associated with cell adhesion. Moreover, the methods of the present invention are useful in treating or inhibiting the pathological conditions where leukocytes and lymphocytes cause cellular or tissue damage.

Through this inhibitory action, the present invention includes methods comprising administering to a mammal in need thereof a therapeutically effective amount of the compound of the present invention to treat the conditions including, but not limited to, asthma, stroke, reperfusion injury, trauma, transplantation rejection, and atherosclerosis. The methods of this invention also include the treatment of autoimmune diseases including, but not limited to arthritis, lupus, multiple sclerosis, Type I diabetes, psoriasis, inflammatory bowel disease, and other inflammatory diseases and conditions.

This invention also comprises pharmaceutical compositions utilizing the compound of this invention. The compound of the present invention may be administered in any manner sufficient to deliver a therapeutic dose, including orally, parenterally or topically. Oral formulations will likely be preferred for most chronic ailments, with parenteral administrations being particularly useful for acute maladies, such as trauma or stroke. Topical formulations may be more desirable for certain autoimmune problems, such as psoriasis. These compounds may be administered neat or with a pharmaceutical carrier to a mammal in need thereof. The pharmaceutical carrier may be solid or liquid.

A solid carrier can include one or more substances which may also act as excipients, flavoring agents, lubricants, solubilizers, suspending or stabilizing agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars such as sucrose, glucose, fructose and confectioner's sugar, lactose, dextrin, dry starch (e.g. corn, potato or tapioca starch), gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredients can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic

solvent, a mixture of both, or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably a sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by, for example, intramuscular, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compound can also be administered orally either in liquid or solid composition form.

Preferably, the pharmaceutical composition is in unit dosage form, e.g. as tablets or capsules. In such form, the composition is subdivided in unit dose containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example, packeted powders, vials, ampoules, prefilled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form. The dosage to be used in the treatment must be subjectively determined by the attending physician.

The dosage requirements will vary with the particular pharmaceutical composition employed, the route of administration, the severity of the symptoms presented and the particular subject being treated. Projected daily dosages of active compound would be from about 0.1μg/kg to about 100 mg/kg, preferably between 0.001-25 mg/kg, and more preferably between 0.01-5 mg/kg. Treatment will generally be initiated with small dosages less than the optimum dose of the compound. Thereafter the dosage is increased until the optimum effect under the circumstances is

reached; precise dosages for oral, parenteral, nasal, or intrabronchial administration will be determined by the administering physician based upon experience with the individual subject treated. Preferably, the pharmaceutical composition is in unit dosage form, e.g. as tablets or capsules. In such form, the composition is sub-divided in unit dose(s) containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example, packeted powders, vials, ampoules, prefilled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form.

In addition, the compounds of this invention may be employed as a solution, cream, or lotion by formulation with pharmaceutically acceptable vehicles containing 0.5-5 percent, preferably about 2%, of active compound which may be administered to an affected area, such a surface area exhibiting the effects of psoriasis.

The following examples demonstrate the ability of the compounds of this invention to selectively inhibit, block or modulating the function of the Beta2 Integrin family of cell adhesion molecules. The specific compound referred to as asperlin in the Examples below is 6,7-Anhydro-2,3,8-trideoxy-D-galacto-oct-2-enoic acid.delta.-lactone 4-acetate, also referred to herein as 5,6-dihydro-5(S)-acetoxy-6(S)-(1,2-trans-epoxypropyl)-2H-pyran-2-one.

EXAMPLE I

Asperlin inhibits the adhesion of activated, β2 integrin-expressing HL60 cells to recombinant soluble ICAM-1

Materials

HL60 cells were provided by the American Type Culture Collection (ATCC No. CCL240) and were used between passages 20 to 30 as the control β2 integrin expressing cells, following stimulation for exactly four days in culture media containing dimethylsulfoxide (DMSO). Culture media was comprised of RPMI 1640 (Gibco No. 3201870AJ) supplemented with Penicillin (100 Units/ml)/ Streptomycin Sulfate (100 mg/ml) (Gibco No. 600-5145AE), L-Glutamine (2mM) (Gibco No. 320-5030PG), and 10% heat inactivated fetal bovine serum (FBS, Hyclone No. A-1111L). Fetal bovine

serum was heat inactivated by incubating in a 56°C water bath for 30 minutes. Stimulation of the expression of the β_2 integrin on HL60 cells was achieved by growing the cells at a density of 2.5 X 10^5 cells per ml of culture media for four days in the presence of 1.25% DMSO. Following the removal of the DMSO containing media, the β_2 integrin on these cells was activated to a high affinity ICAM-1 binding state by the addition of 0.1nM of (PMA) and PMN Buffer. This buffer was comprised of Hank's balanced salt solution (HBSS) supplemented with 1.2mM calcium chloride, 1mM magnesium chloride, 2% glucose, 20μM HEPES buffer. The ICAM-1 used to measure the adhesion of β_2 integrin-bearing cells was obtained from cloning and expression of recombinant, soluble, human ICAM-1 using a baculovirus expression system and standard molecular biology technology. Soluble ICAM-1 was cloned by cleaving ICAM-1 DNA, purchased from R & D Systems, with restriction enzymes to obtain DNA that codes for the soluble (non-membrane) form of the protein. This DNA was then cloned into a baculovirus vector and the expression of soluble ICAM-1 was achieved in Sf9 cells using a kit obtained from Invitrogen Corporation. The soluble ICAM-1 was purified by passing the media from the ICAM-1 expressing cells over an anti-ICAM-1 antibody-linked sepharose column prepared using a standard immunoaffinity chromatography kit obtained from Pierce Inc.

Procedure

200ng of purified, recombinant, soluble ICAM-1 contained in 100μl of PBS was added to wells of a flat bottom, 96 well EIA/RIA plate (Corning No. 25801), covered with an adhesive backed plate sealer (Linbro No. 76-401-05) and incubated for at least 18 hours at 4°C to allow ICAM-1 to bind to the assay wells, prior to the addition of the β_2 integrin expressing HL60 cells.

HL60 cells were grown in 1.25% DMSO for 4 days and pelleted by centrifugation in a Sorvall RT6000 tabletop centrifuge for 5 minutes, 1000 rotations per minute (rpm), at room temperature. The resulting HL60 cell pellet was resuspended in 50ml sterile Dulbecco's phosphate buffered saline (dPBS) (Gibco No. 310-4040AJ). The resulting cell pellet was resuspended in 50 ml dPBS and the cell concentration was determined using a hemacytometer. The cells were pelleted as described above and resuspended in PMN Buffer to 15 to 20 X 10^6 cells/ ml. The resuspended HL60 cells

were fluorescently labeled by mixing the cells with an equal volume of a 25μM solution of Calcein AM (Molecular Probes No. C-1430) dye that had been dissolved in PMN Buffer. HL60 cells in the calcein AM solution was incubated in a 37°C waterbath for 10 minutes with intermittent swirling of the reaction tube. The labeling reaction was stopped by the addition of 13 ml of ice-cold PMN Buffer and the cells were pelleted by centrifugation at 2000 rpm for 5 minutes at 4°C.

The labeled cell pellet was resuspended in 15ml of ice-cold PMN Buffer and the cell density was determined using a hemacytometer. Cells were then pelleted by centrifugation at 2000 rpm for 5 minutes followed by resuspension in ice-cold PMN Buffer to 2 $X10^5$ cells/ 40μl. The labeled cell suspension was placed in the dark at room temperature while asperlin containing solutions were prepared.

Asperlin was solubilized in 100% DMSO at a concentration of 50mg/ml. This stock was then diluted to 400μg/ml with PMN Buffer and serial dilutions of the 400μg/ml asperlin solution were prepared using PMN buffer containing 2% DMSO to obtain solutions ranging from 200μg /ml to 1.56μg/ml.

The ICAM-1 assay plate was removed from 4°C and allowed to warm to room temperature and non-adherent ICAM-1 was aspirated from the assay plate using a multichannel pipettor. 200μl of 1% Tween-20 in dPBS was added to the ICAM-1 coated assay wells and the plate was incubated for exactly 2 minutes at room temperature. The 1% Tween-20/dPBS solution was removed from the wells by inverting the assay plate and shaking out the liquid. Wells of the assay plate were washed 4X with 200μl/well of PMN Buffer. After each wash, the plate was inverted and blotted on paper toweling to remove excess liquid. 50μl of the serially diluted asperlin was added to wells of the assay plate. The control wells received 50μl of PMN buffer containing 2% DMSO and the assay plate was incubated at room temperature for 10 minutes.

Calcein labeled cells, that had been gently mixed by swirling, were added to the wells in 40μl volumes equal to 2 X 10^5 cells/ well, followed immediately by addition of 10μl of $1x10^{-6}$M PMA solution to all assay wells using a multichannel pipettor. The

well contents were mixed using the same multichannel pipettor and the assay plate was incubated for 30 minutes in a 37°C, 5% CO_2, humidified incubator.

The assay plate was removed from the 37°C incubator and total fluorescence of the labeled ß2 integrin expressing cells in each well of the assay plate was measured using a fluorescent microtiter plate reader (Cytoflour, Millipore Corp). Non-adherent cells were aspirated from the wells of the assay plate using a multichannel pipettor. Wells of the assay plate were washed 3X with 200µl PMN buffer/ well. 100µl of PMN buffer was added to wells of the assay plate. The fluorescence of the adherent, activated, ß2 integrin expressing cells was measured using the fluorescent plate reader, as just described.

The percentage of ß2 integrin expressing cells adhering to ICAM-1 wells was quantitated by the following equation:

% Cells Bound = (Bound Cell Fluorescence ÷ Total Cell Fluorescence) X 100

The percentage of inhibition of ß2 integrin expressing cells adhering to ICAM-1 in the presence of asperlin was quantitated by the equation: % Inhibition of Cell Adhesion = 100% - [(Average % Cells Bound in the presence of asperlin ÷ Average % Cells Bound in control wells) X 100]

The results obtained in this experiment, shown in the following table (Table I), demonstrated that asperlin blocks the adhesion of activated ß2 integrin expressing cells to ICAM-1. The control wells, that do not contain asperlin, represent 0% inhibition in binding of ß2 integrin expressing cells to ICAM-1. This suggests that asperlin is an inhibitor of ß2 integrin mediated adhesion to ICAM-1.

TABLE I

Asperlin µg/ml	Total Cell Fluorescence (fluorescence units/well)	Bound Cell Fluorescence (fluorescence units/well)	Percent Cells Bound	Average Percent Cells Bound	% Inhibition of Cell Adhesion
200	5764	29	0.5	0.5	98
	5796	28	0.5		
100	6504	45	0.7	0.6	97.5
	6377	31	0.5		
50	5764	82	1.4	1.4	94
	5829	77	1.3		
25	6522	171	2.6	4	83
	6080	320	5.3		
12.5	6029	589	10	12	50
	6829	768	13		
6.25	6541	1003	15	17	29
	6218	1161	19		
3.125	5747	1862	32	33	-36
	6166	2077	34		
1.56	6236	1551	25	26	-8
	6468	1755	27		
0, Control	5961	1231	21	24	
	5796	1433	25		
	5651	1129	20		
	6149	1750	28		
	6063	1740	29		
	5731	1073	19		

EXAMPLE II

Asperlin does not inhibit the adhesion of activated, ß1 Integrin -expressing U937 cells to human fibronectin

Materials

U937 cells, a human monocyte-like, histiocytic lymphoma cell line, was acquired from American Type Culture Collection (ATCC No. CRL-1593). The cells were grown in the culture media described in Example I, materials. Cells were subcultured when the cell density was approximately $1x10^6$ cells per ml. The ß1 integrins on U937 cells were activated to bind to fibronectin by the addition of PMN Buffer, described in Example I. The human fibronectin (Gibco, No. XOO1) was diluted to 3.5µg/ml with dPBS. A 1% Bovine Serum Albumin (BSA; Fraction V, ICN Corp., No. 810032) solution was prepared in dPBS and was sterile filtered using a 0.22mM disposable filter apparatus (Corning No. 25932-200) before use. The 1% BSA solution was used for blocking non-specific binding sites on the plastic wells of the assay plate. The assay plates used were Corning 96-well EIA/RIA plates (No. 25801).

Procedure

350ng of human fibronectin contained in 100µl of dPBS was added to wells of the assay plate. The control wells were filled with dPBS only. The assay plate was incubated at room temperature for exactly two hours to allow the fibronectin to bind to the assay wells. The fibronectin solution was aspirated from the wells of the assay plate and the wells were washed 3X with 200µl/ well of dPBS. The assay wells were filled with the 1% BSA solution. Additional wells, not coated with fibronectin, were also filled with the 1% BSA solution; these wells were used to measure non-specific cell adherence to the plastic assay plate. The assay plate was incubated for 30 minutes at room temperature. The 1% BSA solution was removed from the wells of the assay plate by aspiration using a multichannel pipettor. The assay wells were washed 3X with 200µl/well of dPBS. Approximately 50µl of dPBS was added to the assay wells to ensure that they would not dry out prior to the initiation of the assay.

U937 cells were harvested from culture, washed and labeled with calcein AM fluorescent dye as outlined in Example I, except that the concentration of calcein used to

label U937 cells was 12.5μM and cells were sedimented by centrifugation at 1000rpm for 5 minutes. After the final wash (see Example I), the U937 cells were resuspended in ice-cold PMN Buffer to 1.25 X 10^5 cells/ 40μl. The cell suspension was placed in the dark at room temperature until needed. The asperlin solutions were prepared as described in Example I.

The 50μl of dPBS remaining in the wells of the assay plate was removed by inverting the assay plate and tapping on paper toweling. 50μl of the serially diluted asperlin solution was added to wells of the assay plate. Control wells received PMN buffer containing 2% DMSO and the assay plate was incubated for 10 minutes at room temperature.

Calcein labeled cells, that had been gently mixed by swirling, were added to the wells in 40μl volumes equal to 1.25 X 10^5 cells/well and 10μl of PMA solution was immediately added to the wells of the assay plate and the assay was continued as described in Example 1. 100μl of PMN buffer was added to all wells of the assay plate. The fluorescence of the adherent, activated β_1 integrin expressing cells was measured using a fluorescent plate reader, as described in Example I.

The percentage of β_1 integrin expressing cells adhering to fibronectin coated wells was quantitated by the equation: % Cells Bound = ((Bound Cell Fluorescence - Average Bound Fluorescence of the Non-specific Binding Control) ÷ Total Cell Fluorescence) x100.

The percentage of inhibition of β_1 integrin expressing cell adhesion to human fibronectin in the presence of asperlin was quantitated, as described in Example I. The results obtained in this experiment, shown in the following table (Table II), demonstrate that asperlin does not block the adhesion of β_1 integrin expressing cells to fibronectin.

TABLE II

Asperlin µg/ml	Total Cell Fluorescence (fluorescence units/well)	Bound Cell Fluorescence (fluorescence units/well)	Percent Cells Bound	Average Percent Bound	% Inhibition Cell Adhesion
200	3019	2222	61	67	3
	3475	2736	68		
	3455	2846	71		
100	3407	2466	61	69	0
	3534	2854	70		
	3738	3194	75		
50	2960	2391	68	73	-6
	3257	2791	74		
	3635	3203	77		
25	3276	2404	61	69	0
	3485	2870	71		
	3455	2969	75		
12.5	3045	2331	64	70	-1
	3248	2683	71		
	3495	2985	74		
6.25	3194	2292	60	62	10
	3248	2344	60		
	3294	2529	65		
3.125	3594	3132	76	75	-9
	3686	3140	75		
	3446	2895	73		

TABLE II (Continued)

Asperlin µg/ml	Total Cell Fluorescence (fluorescence units/well)	Bound Cell Fluorescence (fluorescence units/well)	Percent Cells Bound	Average Percent Bound	% Inhibition Cell Adhesion
1.56	3465	3002	75	75	-9
	3645	3140	76		
	3697	3132	74		
0, Control	2696	2344	66	69	
	2919	2318	66		
	3105	2384	64		
	3436	2846	72		
	3379	2799	71		
	3322	2646	68		
	3717	3221	76		
	3455	2838	71		
	3294	2572	66		
Non-Specific Binding	3584	378			
	3370	328			
	3350	339			
	3341	409			
	3239	405			
	2744	461			

EXAMPLE III

Asperlin does not inhibit the adhesion of HL60 Cells to E-selectin

The following experiments demonstrate that asperlin does not affect the binding of HL-60 cells to recombinant, soluble, human E-selectin (rsE-selectin).

Materials

rsE-selectin was prepared and purified using standard molecular biology techniques. HL60 cells were obtained from American Type Culture Collection (ATCC) and maintained in culture media, as described in Example I, except that cells could be used in the assay at any passage number. Other materials required for the assay are as described in Examples I and II.

Procedure

200ng of purified, recombinant, soluble E-selectin contained in 100µl of dPBS was added to wells of a flat bottom 96 well EIA/RIA plate. The control wells were filled with dPBS only and the assay plate was incubated at 4°C for at least 18 hours to allow the rsE-selectin to bind to assay wells. The rsE-selectin solution was removed from the wells of the assay plate, washed, and blocked with 1% BSA, as described in Example II.

HL60 cells were harvested from culture, washed and labeled with calcein AM as outlined in Example I, except that the concentration of calcein used to label the HL-60 cells was 12.5µM and cells were centrifuged at 1000rpm for 5 minutes. After the final cell wash (see Example I), the cells were resuspended in ice-cold PMN buffer to 1.0 X 10^5 cells / 40µl and the cells were placed in the dark at room temperature until added to the assay plate.

The asperlin solutions were prepared as described in Example I. The dPBS remaining in the wells of the assay plate was removed by inverting the plate and tapping it on paper toweling and the assay was performed as described in Example II. 100µl of PMN buffer was added to all wells of the assay plate and the fluorescence of the adherent HL-60 cells binding to rsE-selectin was measured using a fluorescent plate

reader, described in Example I. The percentage of HL60 cells binding to E-selectin and the percentage of inhibition of HL60 cell adhesion to E-selectin in the presence of asperlin were also quantitated as described in Example II.

The results obtained in this experiment, shown in the following table (Table III), indicate that asperlin does not inhibit E-selectin mediated HL60 cell binding.

TABLE III

Asperlin µg/ml	Total Cell Fluorescence (fluorescence units/well)	Bound Cell Fluorescence (fluorescence units/well)	Percent Cells Bound	Average Percent Bound	% Inhibition
200	1720	1124	52	48	8
	1478	887	44		
100	1832	1412	64	62	-19
	1457	1096	59		
0, Control	1526	977	49	52	
	1544	1017	51		
	1474	953	50		
	1559	1118	57		
	1554	1105	56		
	1637	1084	52		
Non-specific	1520	128			
Binding	1375	89			
	1351	199			
	1650	225			
	1647	270			
	1760	479			

EXAMPLE IV

MTT Cytotoxicity Assay

This experiment describes the affect of asperlin on cellular respiration (mitochondrial activity) by measuring the reduction of the tetrazolium salt MTT to formazan crystals (Moseman, et al). This assay served as a control to ensure that the decrease in the fluorescence observed in Example I, Table I, was not the result of the fluorescent dye leaking from the cells due to disruption of the cell membrane. To address this issue, the mitochondrial activity of β_2 and β_1 integrin expressing cells in the presence of asperlin was quantitated using a commercially available MTT cytotoxicity kit (Promega No. G4100).

Materials

HL60 and U937 cells were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 culture media containing 10% heat inactivated fetal bovine serum (refer to Example I, materials). HL60 cells were used between passages 20 and 30. HL60 cells were stimulated by a four day exposure to DMSO, as described in Example I, materials. Falcon round bottom tissue culture plates (No. 3077) were used for the experiment. All remaining reagents were supplied in Promega's MTT cytoxicity kit (Catalog No. G4100).

Procedure

The assay procedure described in the Promega MTT kit was used with the following changes.

Asperlin was solubilized in 100% DMSO at a concentration of 50mg/ml. The 50mg/ml asperlin stock was diluted to 400µg/ml with culture media containing 5% fetal bovine serum (FBS). This solution was further diluted to 80 and 20µg/ml with culture media supplemented with 5% FBS plus 2% DMSO. 50µl of the 20 and 80µg/ml asperlin solutions were added to wells of a sterile, round bottom 96 well assay plate. Control wells received 50µl of culture media containing 5% FBS plus 2% DMSO. Duplicate assay wells were prepared such that U937 and HL60 cells could be evaluated

on the same plate. The cell density of β_1 integrin expressing U937, and β_2 integrin expressing HL60 cell cultures were determined using a hemacytometer.

Cells were harvested by centrifuging for 5 minutes at 1000 rpm, room temperature, in a Sorvall RT600 bench-top centrifuge. The HL60 cell pellet was resuspended in fresh culture media at a concentration of $5x10^6$ cells/ml. The U937 cell pellet was resuspended in fresh culture media at a concentration of $2.5x10^6$ cells/ml. $2.5x10^5$ β_2 integrin expressing HL60 cells, contained in 50µl, were added to wells of the assay plate using a multichannel pipettor. In duplicate assay wells, $1.25x10^5$ β_1 integrin expressing U937 cells, contained in 50µl, were added to wells of the assay plate using a multichannel pipettor. Assay plates were incubated for either 30 minutes, 60 minutes, or 4 hours in a 37°C, 5% CO_2, humidified incubator.

At the appropriate time point, 15µl of MTT reagent, supplied in the Promega assay kit, was added to each well using a multichannel pipettor. Assay plates were incubated in the 37°C incubator, as just described, for two hours. The MTT reduction reaction was quenched, and the visible formazan crystals were solubilized by adding exactly 100µl of Promega's solubilization buffer to each well. Assay plates were incubated for at least 18 hours in the same 37°C incubator just described to solubilize the formazan reagent. Assay plates were removed from the incubator and allowed to cool to room temperature. The optical density (OD) of each well of the assay plate was determined using a microtiter plate reader (Flow Labs) set at a wavelength of 580nm with a correction wavelength of 630nm.

Replicate sample ODs were averaged. The percentage of inhibition of cellular respiration (mitochondrial activity) by asperlin was quantitated by the following equation:

$$\% \text{ Inhibition} = \left(1-\left(\text{Sample OD} \div \text{Average Control OD}\right)\right) X\ 100$$

The results obtained in this experiment, shown in the following table (Table V), show that asperlin is not cytotoxic to either β_2 or β_1 integrin expressing cells and, the reduction in β_2 integrin mediated cell adhesion by asperlin is not the result of an effect of asperlin on HL60 cellular function.

TABLE V

Asperlin µg/mL	Cell Type	Time Point	Optical Density	Average Optical Density	% Inhibition MTT Reduction
40	HL60	30 min.	.650 .646 .650	.649	27
10	HL60	30 min.	.819 .823 .815	.819	8
0, Control	HL60	30 min.	.942 .889 .891 .897 .875 .873	.895	
40	U937	30 min.	1.362 1.352 1.391	1.386	11
10	U937	30 min.	1.410 1.601 1.584	1.532	0
0, Control	U937	30 min.	1.525 1.551 1.509 1.484 1.550 1.582	1.534	

EXAMPLE V
Asperlin inhibits β2 integrin/ ICAM-1 homotypic binding

The following experiment describes the inhibition of homotypic (cell:cell) binding of β2 and ICAM-1 expressing cells by asperlin. 8866 cells, a human B-cell line, were supplied by Athena Neurosciences (San Francisco, CA). These cells constitutively express both the β2 integrin referred to as LFA-1, and ICAM-1 on the cell surface. In culture, 8866 cells spontaneously bind, or clump together, as a result of β2 integrin binding to ICAM-1. Cell adhesion blocking monoclonal antibodies against LFA-1 have been shown to completely block the binding of β2 integrin to ICAM-1, in the interaction referred to as cell:cell homotypic binding (Rothlein, et al.).

Materials

8866 cells were maintained in RPMI 1640 culture media (refer to Example I) supplemented with 10μM HEPES buffer (Gibco No. 15630-080). Cells were subcultured when the cell density was approximately $1x10^6$ cells/ml. The anti-LFA-1 monoclonal antibody IOT16 (AMAC), was included in each experiment as a positive control inhibitor of the homotypic binding of LFA-1 to ICAM-1.

Procedure

8866 cells were harvested by centrifuging 5 minutes, at 1000 rpm, room temperature, in a Sorvall RT6000 centrifuge. The cell pellet was resuspended in culture media and cells were counted using a hemacytometer. Cells were re-sedimented by centrifuging 5 minutes, at 1000 rpm, room temperature, in a Sorvall RT6000 centrifuge. The cell pellet was resuspended in culture media at a concentration of $2x10^6$ cells/ml. $2x10^5$ cells contained in 100μl were transferred to each well of a flat bottom, 96 well, tissue culture plate (Falcon No. 3072).

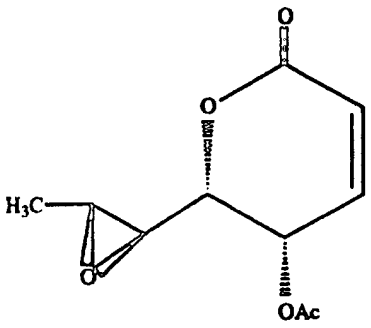
Asperlin was solubilized in 100% DMSO at a concentration of 20mg/ml, 10mg/ml, 5mg/ml, and 2.5mg/ml. These stocks were diluted with culture media to a concentration of 400μg/ml, 200μg/ml, 50μg/ml, and 25μg/ml, respectively. Asperlin was added to wells of the assay plate in volumes of 50μl per assay well. 50μl of culture media containing 2% DMSO was added to control wells of the assay plate. The

monoclonal antibody IOT16 was diluted to 20μg/ml using culture media containing 2% DMSO. 50μl of the 20μg/ml antibody solution was added to positive inhibition control wells on the assay plate. A 200ng/ml solution of PMA was prepared using culture media, and exactly 50μl of this solution was added to wells of the assay plate and the assay plate was incubated in a 37°C, 5% CO_2, humidified incubator for two hours. The progression of homotypic binding was monitored by microscopic observations after 1 and 2 hours of incubation.

Inhibition β_2 integrin dependent homotypic binding to ICAM-1 by asperlin was photographically recorded using a Nikon 35mm camera attached to a Nikon Diaphot 300 inverted microscope (filter setting NCBII, objective 10X, and light setting of photo). Visual inspection of the assay plate after 1 hour of incubation indicated that the monoclonal antibody IOT16 inhibited 8866 cell homotypic binding 100% (cells were not touching or binding to each other). Asperlin was visually observed to also inhibit cell-cell binding in a dose dependent manner. Visual inspection of the assay plate after 2 hours of incubation indicated that the monoclonal antibody IOT16 completely inhibited (100%) 8866 cell homotypic binding. Asperlin inhibited 8866, β_2 integrin mediated homotypic binding to ICAM-1 in a dose dependent manner. These results confirm the effects of asperlin as a blocker of β_2 integrin-mediated homotypic (cell:cell) adhesion.

What is Claimed:

1. A method for treating in a mammal a pathological condition in which leukocytes and lymphocytes cause cellular or tissue damage, the method comprising administering to a mammal in need thereof a therapeutic dosage of a compound of the formula:

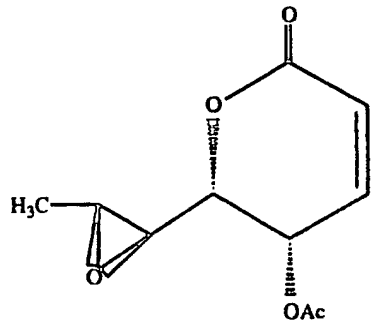


2. The method of Claim 1 wherein the mammal is a human.

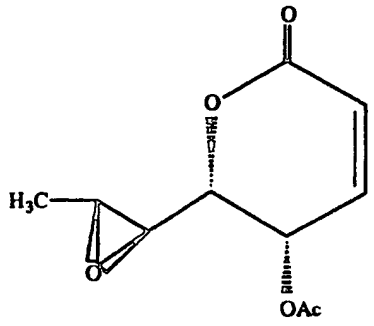
3. The method of Claim 1 wherein the pathological condition is atherosclerosis.

4. The method of Claim 1 wherein the pathological condition is transplantation rejection.

5. A method for treating inflammation in a mammal, the method comprising administering to a mammal in need thereof a therapeutic dosage of a compound of the formula:

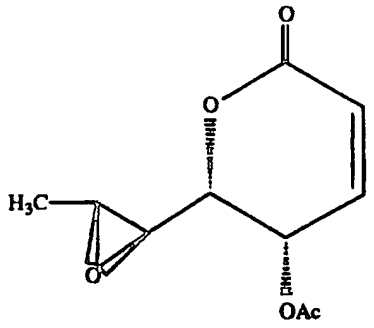


6. A method for inhibiting in a mammal pathological responses associated with intercellular adhesion mediated by the β_2 Integrin family of cell surface molecules, the method comprising administering to a mammal in need thereof a therapeutic dosage of a compound of the formula:



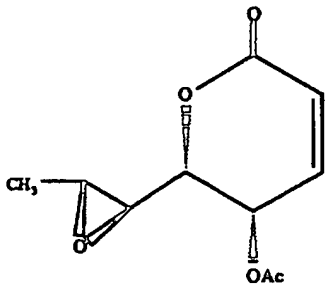
7) The method of Claim 6 in which the pathological response associated with intercellular adhesion are asthma, stroke, reperfusion injury, trauma, transplantation rejection, arthritis, lupus, multiple sclerosis, Type I diabetes, psoriasis, inflammatory bowel disease or atherosclerosis.

8) A pharmaceutical composition which comprises a compound of the structure:



and a pharmaceutically acceptable carrier.

9) The use of a compound of the Formula



in the preparation of a medicament for the treatment of:

a) a pathological condition in which leukocytes and lymphocytes cause cellular or tissue damage;

b) inflammation;

or

c) pathological responses associated with intercellular adhesion mediated by the β_2 Integrin family of cell surface molecules.

INTERNATIONAL SEARCH REPORT

Intern .ial Application No
PCT/US 97/14478

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OWEN: "Biological properties od a new antibiotic, U-13,933" ANTIMICROB. CHEM. CHEMOTHER., vol. 5, 1965, pages 804-807, XP002048388 see the whole document -----	1-9

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
27 November 1997	04. 02. 98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Gac, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/14478

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FR M PCT/ISA/ 210

Remark : Although claims 1-7
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.